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User's Guide

Requirements for Exemption of Regulated Articles

United States Department of Agriculture
Animal and Plant Health Inspection Service
Biotechnology, Biologics, and Environmental Protection

United States
Department of
Agriculture

Animal and
Plant Health
Inspection
Service

Biotechnology,
Biologics, and
Environmental
Protection

Dear Reader:

The purpose of this User's Guide is to help you prepare a petition for exemption for a genetically modified organism subject to the Animal and Plant Health Inspection Service regulations in 7 CFR Part 340. These regulations are entitled, "Introduction of Organisms and Products Altered or Produced Through Genetic Engineering Which Are Plant Pests or Which There is Reason to Believe Are Plant Pests."

While the petition process in § 340.4 of the regulations may be used as a mechanism for either adding organisms to the list of regulated organisms in § 340.2(a) or adding to the list of exempted organisms in § 340.2(b), we anticipate that most petitions submitted will be to expand the list of exempted organisms.

We have included a hypothetical petition for cotton which has been genetically modified to be tolerant to the herbicide glyphosate or resistant to certain lepidopteran insects. It should be noted that the scope and breadth of a potential exemption is determined by the petitioner and the data that is available to support a specific petition for exemption. Inclusion of this petition in the guide is for illustrative purposes only and does not mean that the U.S. Department of Agriculture has already reached a decision on the merits of a petition for this organism or any other organism.

If you have any questions, please call our Biotechnology Permits staff at Area Code (301) 436-7612.

Sincerely,

Terry L. Medley, J.D.
Director
Biotechnology, Biologics,
and Environmental Protection

Table of Contents

Section 1. Questions and Answers

1. How can genetically modified organisms be exempted from the permit requirement under 7 CFR 340?

Submit a petition for an organism to be listed in § 340.2(b), exempted organisms, in accordance with the procedures in § 340.4.

2. What organisms has APHIS already exempted from its regulations?

A permit is no longer required for the interstate movement of *Escherichia coli, Saccharomyces cerevisae, Bacillus subtilis,* or the plant *Arabidopsis thaliana* containing select nucleotide sequences from plant pests, as long as certain biological and physical containment conditions are met.

3. What information is needed in the petition?

The petition must include data proving that a regulated article is not a plant pest and that its introduction would not be harmful to the environment. The petition should include scientific literature that the petitioner is relying on, copies of unpublished studies, or data from tests performed with the modified cultivar. The petition should also include any information that would be unfavorable to the petition for exemption. Do **not** include trade secret or confidential business information since APHIS will make all data submitted in a petition publicly available.

4. How narrow or broad in scope can a petition be? For example, can an exemption for a cultivar be given for a particular geographical region of the country, can I use an exempted cultivar in a breeding program without APHIS review, can I exempt several related gene constructs concurrently (e.g., the coat proteins from all potato virus Y strains)?

The scope of the petition is determined by the petitioner. For example, the petitioner should state explicitly whether he or she intends to use the cultivar in a breeding program, introduce it in only a specific area of the country, or exempt the coat protein gene of all known potato virus Y strains concurrently. Petitioners must submit data which supports the scope of the proposed exemption. Decisions will be made on a case-by-case basis.

5. How will APHIS make the petition for exemption publicly available?

Once a petition is complete, APHIS will publish a notice in the Federal Register notifying the public that the agency has received a petition to exempt an organism. The public will be given an opportunity to comment on the data submitted.

6. How will APHIS inform the States of the petition for exemption?

APHIS will send copies of the Federal Register notice announcing receipt of a petition and the opportunity to comment to all State agriculture regulatory officials.

7. How will comments be addressed in the course of the petition process?

All relevant comments submitted in response to a petition to amend the list of exempted organisms will be addressed in a preamble published in the Federal Register. APHIS expects the comments submitted will provide valuable information for determining the outcome of a petition. A Federal Register notice will announce the final action that APHIS is taking in response to a petition.

8. How can I obtain a copy of the petition?

To request a petition, send a written request to:

Biotechnology Permits
USDA, APHIS, BBEP
6505 Belcrest Road, Room 843
Hyattsville, MD 20872

Requests can be faxed to the above address at Area Code (301) 436-8669.

9. How will APHIS coordinate its review with other Federal agencies?

APHIS will advise other Federal agencies having regulatory jurisdiction over the organism that a petition has been received. This enables the other Federal agencies to advise both APHIS and the petitioner which data in the petition (if any) could be used by the other agencies in their review.

10. How long will it take before APHIS makes a decision on a petition?

APHIS will reach a decision within 180 days after receipt of a complete petition.

11. If APHIS grants an exemption for a gentically modified organism, can it be conditional?

Yes. Whether or not the exemption is conditional depends on the scope of the petition as stated by the petitioner, the data presented, other scientific information, and public comments. For example, in order to prevent the outcrossing of the modified plant with wild relatives, APHIS may impose conditions which would stipulate that the engineered plant be located a minimum distance from certain other plants or that it not be planted within a particular geographical area.

12. Can APHIS revoke or modify an exemption once approved?

Yes. If at some later time scientific data is submitted that suggests the exempted organism has unexpected plant pest characteristics, APHIS can revoke or modify the previous exemption.

13. Once a cultivar has been exempted from APHIS review, do I need a permit to export this cultivar to other countries?

Currently, foreign countries require phytosanitary certificates for the importation of plants and organisms capable of propagation.

The exporting of **all** tobacco plants is regulated by the Marketing Information and Program Analysis Branch of the Tobacco Division of the Agricultural Marketing Service in Washington, DC, at Area Code (202) 447-3489. In addition, the export of certain genetically engineered organisms to specific countries requires a license from the U.S. Department of Commerce, Bureau of Export Administration, Washington, DC, at Area Code (202) 377-5695.

Section 2. Sample Transmittal Letter and Petition Form

Mail Letter and Petition Form

Sample Letter of Transmittal for

Petition Under 7 CFR 340.4

_____, Director
Biotechnology, Biologics, and Environmental Protection
USDA, Animal and Plant Health Inspection Service
6505 Belcrest Road, Room 850
Hyattsville, MD 20782

Dear_____:

Enclosed is a copy of a petition for exemption from regulation of _____ (list organism to be exempted) which has been modifed to_____ (explain the intended effect of the modification). Based on the data contained in the petition, I am requesting that the organism be listed as an exempted organism in 7 CFR section 340.2(b).

The enclosed petition has been prepared in accordance with the provisions of 7 CFR section 340.4 and does not contain confidential business information.

Any questions concerning this petition should be directed to_____ at Area Code ( ) ___-____.

Sincerely,

Sample Cover Page for Petition

Petition to Amend 7 CFR 340.2(b)

The undersigned submits this petition under 7 CFR 340.4 to request the Director, Biotechnology, Biologics, and Environmental Protection to amend the regulations in 7 CFR 340 by adding the following organism: _____ (name of organism to be exempted) to the list of exempted organism in section 340.2(b) of the regulations.

Certification

The undersigned certifies, that to the best knowledge and belief of the undersigned, this petition includes all information and views on which the petitioner relies and that it includes representative data and information known to the petitioner which are unfavorable to the petition.

(Signature) _____
(Name of petitioner) _____
(Mailing address) _____
(Telephone number) _____

Section 3. 7 CFR 340.4

REGULATION

§340.4 Petition to amend the list of organisms.

(a) *General.* Any person may submit to the Director of Biotechnology, Biologics, and Environmental Protection (the Director) a petition to amend the list of organisms in § 340.2 of this part by adding or deleting any genus, species, or subspecies. A petitioner may supplement, amend, or withdraw a petition in writing without prior approval of the Director and without prejudice to resubmission at any time until the Director rules on the petition. A petition to amend the list of organisms shall be submitted in accordance with the procedures and format specified by this section.

(b) *Submission procedures and format.* A person shall submit two copies of a petition to the Director of Biotechnology, Biologics, and Environmental Protection, Animal and Plant Health Inspection Service, U.S. Department of Agriculture, Federal Building, Room 850, 6505 Belcrest Rd., Hyattsville, Maryland 20782. The petition should be dated and structured as follows:

Petition to Amend 7 CFR 340.2

The undersigned submits this petition under 7 CFR 340.4 to request the Director (to add the following genus, species, or subspecies to the list of organisms in 7 CFR 340.2) or (to remove the following genus, species, or subspecies from the list of organisms in § 340.2).

A. Statement of Grounds

A person must present a full statement explaining the factual grounds why the genus, species, or subspecies to be added to § 340.2 of this part is a plant pest or why there is reason to believe the genus, species, or subspecies is a plant pest or why the genus, species, or subspecies sought to be removed is not a plant pest or

PREAMBLE

Petition to Amend the List of Organisms (§ 340.4)

42. Several commenters suggested that USDA should include a mechanism which would allow persons to petition for the "delisting" or removal of organisms from the list of organisms in § 340.2 of the final rule, if it could be demonstrated that such organisms are not plant pests. Other commenters indicated that USDA should include a mechanism that would allow a person to seek the addition of organisms to the list, if it could be shown that such organisms were plant pests.

USDA agrees with the commenters and has added a new § 340.4 to the final rule, entitled "Petition to Amend the List of Organisms." USDA believes that the petition mechanism will afford interested persons the opportunity to readily bring information to USDA's attention as new information becomes available about existing or newly discovered organisms. The petition process in § 340.4 is in accord with section 4(e) of the Administrative Procedure Act (5 U.S.C. 553(e)) for the issuance, amendment, or repeal of a rule and with USDA's Departmental Proceedings in 7 CFR 1.28.

Under § 340.4(a) of the final rule, any person may submit a petition to the Director to amend the list or organisms in § 340.2 by adding or removing any genus, species, or subspecies. Section 340.4(a) further provides that a petitioner may supplement, amend, or withdraw a petition, in writing, without prior approval of the Director and without prejudice to resubmission at any time, until the Director rules on the petition.

Section 340.4(b) specifies the submission procedures and format of a petition. This section requires that a petitioner provide two copies of a petition to the Director.

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why there is reason to believe the genus, species, or subspecies is not a plant pest. The petition should include copies of scientific literature which the petitioner is relying upon, copies of unpublished studies, or data from tests performed. *The petition should not include trade secret or confidential business information.*

A person should also include representative information known by the petitioner to be unfavorable to a petition for listing or delisting. (If a person is not aware of any unfavorable information, the petition should state "Unfavorable Information: NONE.")

B. Certification

The undersigned certifies that to the best knowledge and belief of the undersigned, this petition includes all information and views on which the petitioner relies and that it includes representative data and information known by the petitioner to be unfavorable to the petition.

(Signature) _____
(Name of petitioner) _____
(Mailing address) _____
(Telephone number) _____

(c) Administrative action on a petition. (1) A petition to amend the list of organisms which meets the requirements of paragraph (b) of this section will be filed by the Director, stamped with the date of filing, and assigned a docket number. The docket number shall identify the file established for all submissions relating to the petition. The Director will promptly notify the petitioner in writing of the filing and docket number of a petition. If a petition does not meet the requirements of paragraph (b) of this section,

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Section 340.4(b) also specifies what must be included in the "Statement of Grounds" of the petition. A person must include a full statement explaining the factual grounds why the genus, species, or subspecies to be added to § 340.2 is a plant pest or why there is reason to believe the genus, species, or subspecies is a plant pest. In the case of a petition to remove a genus, species, or subspecies from the list, a person must include a full statement explaining why the genus, species, or subspecies is not a plant pest or why there is no reason to believe the genus, species, or subspecies is a plant pest. The petition should include copies of scientific literature which the petition is relying upon, copies of unpublished studies, or date from tests performed. Because the petition and any accompanying data will be made available for public inspection, the petition should not include trade secret or confidential business information.

A person must also include in the "Statement of Grounds" representative information known to the petitioner which would be unfavorable to a petition to add or remove organisms. Section 340.4(b) also requires that a petitioner sign a short certification that must be included as part of the petition.

Section 340.4(c) specifies the administrative action that will be taken on a petition. Under § 340.4(c), a petition which appears to be complete will be filed by the Director, stamped with the date of filing, and assigned a docket number. The Director will notify the petitioner in writing of the filing and the docket number of the petition. If a petition is incomplete, the petitioner shall be sent a notice indicating how the petition is deficient.

After a complete petition is filed, USDA shall publish a proposal in the Federal Register to amend § 340.2 and solicit comments

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the petitioner shall be sent a notice indicating how the petition is deficient.

(2) After the filing of a petition to amend the list or organisms, USDA shall publish a proposal in the Federal Register to amend § 340.2 and solicit comments thereon from the public. An interested person may submit written comments to the Director on a filed petition, and the comments shall become part of the docket file.

(3) The Director shall furnish a response to each petitioner within 180 days of receipt of the petition. The response will either (i) approve the petition in whole or in part in which case the Director shall concurrently take appropriate action (publication of a document in the Federal Register amending § 340.2 of this part; or (ii) deny the petition in whole or in part. The petitioner shall be notified in writing of the Director's decision. The decision shall be placed in the public docket file in the office of the Director and published in the form of a notice in the Federal Register.

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thereon from the public. Any written comments submitted shall become part of the docket file. The Director shall furnish a written response to each petitioner within 180 days of the receipt of the petition. The decision shall be placed in the public docket file in the office of the Director.

The response will either: (1) approve the petition in whole or in part, in which case the Director shall concurrently take appropriate action (publication of a document in the Federal Register amending § 340.2 of this part); or (2) deny the petition in whole or in part.

APHIS has chosen 180 days as the time period in which to respond to a petition for the following reasons: (1) a 180 day review period would provide APHIS reviewers sufficient time to perform thorough and comprehensive research on the material presented in a petition and to consult with other scientists at other institutions both domestically and internationally; (2) a 180 day review period provides APHIS with sufficient time to schedule public hearings during the petition process should that be necessary; and (3) a 180 day review period is consistent with the petition procedures utilized by other Federal agencies, namely, the Food and Drug Administration in their regulations in 21 CFR 10.30.

Section 4. Sample Petition for Genetically Modified Cotton Plants

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Comments about the guide or issues that may need to be addressed in that section are enclosed in starred boxes.

This guide has been prepared to assist persons in preparing an application for exempting an organism from 7 CFR 340. The guide was prepared as a petition to exempt two cotton cultivars, one which is resistant to select lepidopteran insects and another which is tolerant to the herbicide glyphosate. Certain liberties were taken when preparing the information and data presented. First, APHIS does **not** intend to review an application that contains two cultivars that are transformed for two different genes. We have taken this short cut in order to illustrate data requirements for these two classes of genes, for which exemption petitions are likely and which raise important issues that APHIS must review. Second, with the agronomic performance data and certain of the molecular biology data liberties were taken to ensure that the guide was a reasonable length and that the reader would be able to ascertain the kind of information APHIS expects to be provided in an application.

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Mr. Terry L. Medley, Director
Biotechnology, Biologics and
Environmental Protection
USDA, APHIS
6505 Belcrest Road, Room 850
Hyattsville, MD 20782

Dear Mr. Medley:

Enclosed is a copy of a petition for exemption from regulation of Gossypium hirsutum L. cultivar "Stoneville 825" which have been modified to be tolerant to the herbicide glyphosate or resistant to two Heliothis species. Based on the data contained in the petition I am requesting that the organisms be listed as an exempted organism in 7 CFR 340.2(b).

The enclosed petition has been prepared in accordance with the provisions of 7 CFR section 340.4 and does not contain confidential business information.

Any questions concerning this petition should be directed to me at Area Code (301) 436-5940 or facsimile number Area Code (301) 436-8669.

Sincerely,

Mr. Len Jackson
JPS Biotechnologies

Petition to Amend 7 CFR 340.2(b)

The undersigned submits this petition under 7 CFR 340.4 to request the Director of Biotechnology, Biologics, and Environmental Protection to amend the regulations in 7 CFR 340 by adding the following organisms:

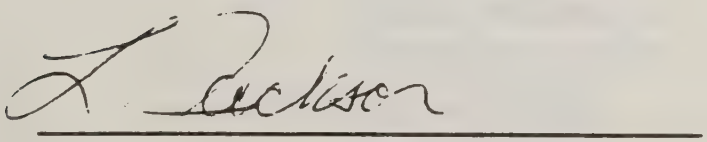
(1) Gossypium hirsutum L. cv. "Stoneville 825LIR" which contains the boll-specific promoter from Gossypium tomentosum, the delta-endotoxin from Bacillus thuringiensis, nopaline synthase termination/ polyadenylation signal sequences from Agrobacterium tumefaciens and the marker gene composed of the nopaline promoter from A. tumefaciens and the neomycin phosphotransferase gene from transposon Tn5.

(2) Gossypium hirsutum L. cv. "Stoneville 825GHS" which contains the nopaline synthase promoter and termination/ polyadenylation signal sequences from A. tumefaciens and a modified 5-enolpyruvyl-3-phosphoshikimate synthase from Salmonella typhyimurium, and the marker gene composed of the nopaline promoter from A. tumefaciens and the neomycin phosphotransferase gene from transposon Tn5,

to the list of exempted organism in section 340.2(b) of the regulations.

Certification

The undersigned certifies, that to the best knowledge and belief of the undersigned, this petition includes all information and views on which the petitioner relies and that it includes representative data and information known by the petitioner to be unfavorable to the petition.



Mr. Len Jackson
JPS Biotechnologies
6505 Belcrest Road
Hyattsville, MD 20782
(301) 436-5940
(301) 436-8669 facsimile

Abbreviation and Scientific Terms

Bollworm	*Heliothis zea*
Bt	delta-endotoxin from *Bacillus thuringiensis*
Cabbage looper	*Trichoplusia ni*
Corn earworm	*Heliothis zea*
Cotton leafworm	*Spodoptera exigua*
Dinoseb	an insecticide
DMSA (herbicide)	monosodium methylarsonic acid
DSMA (herbicide)	disodium methylarsonic acid
EPSP synthase	5-enolpyruvyl-3-phosphoshikimate synthase
European corn borer	*Ostrinia nubilalis*
Fluazifop (herbicide)	Fusalide®
Fluometuron (herbicide)	Cotoran®
Glyphosate (herbicide)	Roundup®
Heliothis zea	bollworm, corn earworm, tomato fruitworm, tobacco false budworm
Heliothis virescens	tobacco budworm
IPM	Integrated Pest Management
NOS	nopaline synthase
NPT II	neomycin phosphotransferase
Ti plasmid	tumor-inducing plasmid
Tn*5*	transposon *5*
Tobacco horn worm	*Manduca sexta*
Tobacco false budworm	*Heliothis zea*
Tobacco budworm	*Heliothis virescens*
Tomato fruitworm	*Heliothis zea*
Trifluralin (herbicide)	Treflan®

OUTLINE OF PETITION

Rationale for Development of the Transformed Plants

JPS Biotechnologies has developed genetically transformed cotton plants which are resistant to two lepidopteran insects - bollworm (Heliothis zea) and tobacco budworm (H. virescens). These major insect pests of cotton are currently controlled by chemical insecticides, usually the pyrethroids. Resistance to pyretheroid insecticides has developed and alternative chemical insecticides are more expensive and less effective than the pyretheroids. Introduction of transformed plants resistant to these lepidopteran insects into current integrated pest management systems for cotton will most likely result in reduced chemical insecticide application or if pyretheroid-resistant Heliothis spp. are present the introduction of a new, more environmentally compatible insect control method.

JPS Biotechnologies also has developed genetically transformed cotton plants which are tolerant to the herbicide glyphosate. The major weed pests of cotton in the southern U. S. include morningglories, cocklebur, pigweeds, johnsongrass, nutsedges, prickly sida, and bermudagrass. The development of glyphosate tolerant cotton will allow producers the option of applying glyphosate postemergence in an over-the-top application or replace preemergence herbicides under appropriate conditions. Introduction of these plants will offer producers several advantages: replacement of more toxic herbicides including arsenic compounds, with a more environmentally-benign herbicide; the introduction of the new plants and herbicide application is compatible with Integrated Pest Management; and glyphosate does not have carry-over problems and restriction on application that some currently approved cotton herbicides have.

II. The Cotton Family

Cotton as a Crop

Four species of the genus Gossypium are known as cotton, which is grown primarily for the seed hairs that are made into textiles. Cotton is predominant as a textile fiber because the mature dry hairs twist in such a way that fine, strong threads can be spun from them. Other products, such as cottonseed oil, cake, and cotton linters are byproducts of fiber production.

Cotton, a perennial plant cultivated as an annual, is grown in the United States mostly in areas from Virginia southward and westward to California, in an area often referred to as the Cotton Belt (McGregor, 1976).

Taxonomy of Cotton

The genus Gossypium, a member of the Malvaceae, consists of 39 species, four of which are generally cultivated (Fryxell, 1984). The most commonly cultivated species, G. hirsutum L. Other cultivated species are G. arboreum L., G. barbadense L., and G. herbaceum L.

Four species of Gossypium occur in the United States (Fryxell, 1979; Kartesz and Kartesz, 1980). Gossypium hirsutum is the primary cultivated cotton. Gossypium barbadense is also cultivated. The other two species, G. thurberi Todaro and G. tomentosum Nuttall ex Seemann, are wild plants of Arizona and Hawaii, respectively. Gossypium tomentosum is known from a few strand locations very close to the ocean.

Genetics of Cotton

At least seven genomes, designated A, B, C, D, E, F, and G, are found in the genus (Endrizzi, 1984). Diploid species (2n=26) are found on all continents, and a few are of some agricultural importance. The A genome is restricted in diploids to two species (G. arboreum, and G. herbaceum) of the Old World. The D genome is restricted in diploids to some species of the New World, such as G. thurberi.

By far, the most important agricultural cottons are G. hirsutum and G. barbadense. These are both allotetraploids of New World origin, and presumably of ancient cross between Old World A genomes and New World D genomes. How and when the original crosses occurred have

been subject to much speculation. Euploids of these plants have 52 somatic chromosomes, and are frequently designated as AADD. Four additional New World allotetraploids occur in the genus, including G. tomentosum, the native of Hawaii. Gossypium tomentosum has been crossed with G. hirsutum in breeding programs.

The New World allotetraploids are peculiar in the genus, because the species, at least in their wild forms, grow near the ocean, as invaders in the constantly disturbed habitats of strand and associated environs. It is from these "weedy" or invader species that the cultivated cottons developed (Fryxell, 1979).

Pollination of Cotton

Gossypium hirsutum is generally self-pollinating, but in the presence of suitable insect pollinators can exhibit cross pollination. Bumble bees (Bombus spp.), Melissodes bees, and honey bees (Apis mellifera) are the primary pollinators (McGregor, 1976). Concentration of suitable pollinators varies from location to location and by season, and is considerably suppressed by insecticide use. If suitable bee pollinators are present, distribution of pollen decreases considerably with increasing distance. McGregor (1976) reported results from an experiment in which a cotton field was surrounded by a large number of honey bee colonies, and movement of pollen was traced by means of fluorescent particles. At 150 to 200 feet, 1.6 percent of the flowers showed the presence of the particles. The isolation distance for Foundation, Registered, and Certified seed in 7 CFR Part 201 is 1320 feet, 1320 feet, and 660 feet, respectively.

Gossypium tomentosum seems to be pollinated by lepidopterans, presumably moths (Fryxell, 1979). The stigma in G. tomentosum is elongated, and the plant seems incapable of self-pollination until acted upon by an insect pollinator. The flowers are unusual too, because they stay open at night; most Gossypium flowers are ephemeral: they open in the morning and wither at the end of the same day.

Weediness of Cotton

Although the New World allotetraploids show some tendencies to "weediness" (Fryxell, 1979), the genus shows no particular weedy aggressive tendencies. Cotton is not allowed to overwinter in the U.S. Cotton is a poor competitor in most of the Southern U.S. cotton growing

regions. in areas where freezing conditions occur, the cotton plant cannot overwinter and there is essentially no volunteerism from seed.

Modes of Gene Escape in Cotton

Genetic material of G. hirsutum may escape from a test area by vegetative material, by seed, or by pollen.

Propagation by vegetative material is not a common method of reproduction of cotton. Physical safeguards that inhibit the movement of vegetative material from the area should be adequate to prevent gene movement by this means. Movement of seed from the test area can likewise be inhibited by adequate physical safeguards.

Movement of genetic material by pollen is possible only to those plants with the proper chromosomal type, in this instance only to those allotetraploids with AADD genomes. In the United States, this would only include G. hirsutum, G. barbadense, and G. tomentosum. Gossypium thurberi, the native diploid from Arizona with a DD genome, is not a suitable recipient. Movement to G. hirsutum and G. barbadense is possible if suitable insect pollinators are present, and if there is a short distance from transgenic plants to recipient plants. Physical barriers, intermediate pollinator-attractive plants, and other temporal or biological impediments would reduce the potential for pollen movement.

Movement of genetic material to G. tomentosum is more unknown. The plants are chromosomally compatible with G. hirsutum, but there is some doubt as to the possibility for pollination. The flowers of G. tomentosum seem to be pollinated by moths, not bees. And they are receptive at night, not in the day. Both these factors would seem to minimize the possibility of cross-pollination. However, Fryxell (1979) reports that G. tomentosum may be losing its genetic identity from introgression hybridization of cultivated cottons by unknown means.

Characteristics of Nontransformed Cultivar

G. hirsutum L. cv. "Stoneville 825" is the cultivar that was genetically transformed. This cultivar is widely grown in the U.S. and was specially developed for introduction in the Mississippi delta region. JPS has received a U. S. patent on the specific delta-endotoxin and herbicide tolerance gene that has been transformed into this cultivar and the transformed Stoneville 825 has

received additional protection under Plant Variety Protection Act of 1970. JPS does not intend to introduce the new traits into other cotton cultivars by traditional breeding techniques.

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The applicant should state whether or not the cultivar to be exempted will be sole cultivar to be considered, whether the cultivar will be used in a traditional breeding program, or whether the genes can be introduced into a specific class of cultivars (e.g., sterile potatoes).
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The cultivar that has been transformed to be herbicide tolerant now called G. hirsutum cv. "Stoneville 825GHS" and the insect resistant line "Stoneville 825LIR". The term GHS stands for glyphosate herbicide tolerant and LIR stands for lepidopteran insect resistant.

III. Description of Transformation System and Regulatory Sequences to be Exempted

Agrobacterium tumefaciens Transformation System

The vector system used to transfer the transformed genes to cotton plants is based on the Ti plasmid from Agrobacterium tumefaciens. The vector system is "disarmed" or nonpathogenic because all the genes involved in phenotypic expression of the disease characteristics have been deleted. We have utilized a "two-component cointegrate" system for transferring genes into cotton. This system was chosen for its use in cloning genes of interest into plasmids and for greater transformation frequencies with certain plant species and/or cultivars and was developed by Deblaere et al. (1985).

CONSTRUCTION OF ACCEPTOR PLASMID: The purpose of this procedure is to obtain a Ti plasmid lacking all of the T-region DNA (phytohormone and pathogenicity genes) but retaining the vir region. The details of the construction of this plasmid has been previously published (DeBlaere et al. 1985). An octopine plasmid pTiB6S3 containing two adjacent T-regions (T_L and T_R) was modified to contain a kanamycin resistance marker. The intermediate vector pGV746, a pBR322 derivative, contains two Ti plasmid sequences that are located respectively to the left and outside the T_L DNA segments and to the right and outside of the T_R DNA sequences. A double recombination between pGV746 and pGV2217 results in pGV2260 (fig. 1). The physical structure of one Rif^R, Cb^R, and Km^S transconjugant, pV2260 was verified by Southern hybridization and the results have been published (Deblaere et al. 1985). In pGV2260, the entire T_L and T_R regions are deleted and substituted by sequences derived from pBR322.

CONSTRUCTION OF THE VECTOR PLASMID: The initial plasmid pGV700 is a pBR322 derivative containing a 1 kb HindIII/BglII fragment derived from the HindIII-18 fragment of pTiAch 5 and the 6.5 Kb BglII/HindIII part of HindIII fragment of pTiACh5 (see fig. 2). This plasmid contains all T-region sequences except genes 5,7,2, and 1. A 7.5 kb HindIII fragment from pGV700 was recloned into pGV600, giving rise to pGV742. pGV600 is a pBR322 derivative lacking any BamHI sites. The remaining T_L DNA sequences (but not T_L 25 bp border sequences) in pGV742 were removed by deleting internal BamHI fragment giving rise to pGV744. The T_R DNA sequences (but not the RB 25 bp sequences) were removed by deleting the internal EcoRI

fragment, giving rise to pGV749. To obtain a plasmid containing only border sequences, the 1.87 kb HindII/NruI fragment from pGV749 was cloned into pGV710, previously digested with EcoRI/HindIII. pGV710 is a pBR322 derivative containing Sm^R, Su^R, Cm^R, and Tc^R markers. The sticky ends obtained after digestion were flush-ended by treatment with Klenow DNA polymerase prior to HindIII treatment. The resulting plasmid pGV815 was isolated as a Sm^R, Cb^R, Cm^S, Tc^S clone. The EcoRI and HindIII sites of this plasmid were eliminated by filling in the sticky ends and self ligation of the vector. The chimeric kanamycin was produced by inserting a 298 bp BclI/BamHI fragment from pGV230 (which contains a NOS promoter) into BclI site of pKC7 to produce pKC7::NOS. Plasmid pCK7 is a pBR322 derivative containing 1.8 kb HindII/Bam HI fragment of Tn5 which carrying the NPT II gene (Rao and Rogers 1979). The chimeric NPT II gene was isolated as a BclII/BamHI fragment and cloned into BglII site of pGV825 to produce pGC831 (DeBlaere et al. 1985). When the chimeric insecticidal protein, delta-endotoxin (Bt), is introduced into pGV831 the plasmid is called pJLW180LIR and when the glyphosate-tolerance gene, EPSP synthase, is inserted into pGV831 the plasmid is designated pJLW180GHS (fig 3).

The disarmed A. tumefaciens transformation system described above and in publications (DeBlaere et al. 1985) does not incite galls (tumors) when inoculated on susceptible plants. The scientific literature supports the view that only the T-region is transferred and integrated into the plant genome (Fraley et al. 1986 Cooper and Meredith 1989) and in this vector system, all T-DNA is deleted.

Description of the NOS Promoter

Strong promoters are DNA sequences located upstream from functional gene sequences. Strong promoters allow high levels of mRNAs to be synthesized which then results in high levels protein production. The promoter used in engineering these plants was derived from the nopaline synthase gene from A. tumefaciens Ti plasmid. This promoter is well-characterized has been used extensively in the development and testing of transgenic plants. The promoter is wound-inducible and auxin-inducible (An et al. 1990) and its expression is organ-specific and developmentally regulated under certain circumstances (An et al. 1988). Host cell factors also can influence gene expression under its direction (Ha and An 1989).

The NOS synthase region was obtained on a 350 bp Sau3A fragment from the HindIII 23 fragment of plasmid pTiT37 (Depicker et al. 1982) and was transformed to remove the entire nopaline synthase coding sequence. The resulting promoter fragment extended from base -264 to base 35 of the nopaline synthase sequence was positioned next to the BglII site located just outside the coding sequence of the EPSP synthase gene for herbicide tolerance.

Although the NOS promoter is derived from known plant pest, it cannot cause any disease by itself nor in conjunction with any sequences located on the disarmed Ti plasmid (Rogers et al. 1985; Nagy et al. 1985). The NOS promoter has been used in hundreds of plant transformations with many different genes without any report of unexpected consequences.

Description of the Boll-specific Promoter

Since bollworms and budworms cause significant damage to cotton bolls (fruits) and squares (flower buds) without causing significant damage to the foliage (Davidson and Lyon 1987), JPS has isolated a promoter from cotton plants specifically active in the insect target tissues. The isolation, characterization, sequencing, of this promoter was previously published (White 1990). This promoter directs the expression of proteins to high levels in carpel walls of the boll tissues and significantly less expression (90%) in the squares. It is nearly inactive in other cotton tissues. This promoter was used to direct the synthesis of Bt protein.

Description of the NOS terminator/polyadenylation sequences

Termination/polyadenylation sequences are sequences located near the 3' end of the transformed gene. Presence of these sequences results in the termination of mRNA synthesis and the addition of a series of adenosine residues required for translation of the mRNA into protein. These regulatory sequences were used for both the Bt and EPSP synthase genes. To isolate the NOS termination sequence a 260 bp Mbo I fragment (base 1,297 to base 1554 of the published sequence (Depicker et al., 1982)), was isolated from the HindIII-23 fragment. This fragment was ligated together with the EcoRI-BamHI fragment that contained the nopaline synthase promoter and engineered gene to yield the intact chimeric gene on a 1.5 kilobase EcoRI fragment.

The specific promoters and NOS termination signal sequences were blunt-end ligated to the cloned genes and inserted into pGV831 at the unique BamH1 site. A map of the resulting plasmid pJLW180 is shown in figure 3.

Agrobacterium-mediated Transformation

Plasmid JLW180GHS or pJLW180LIR were introduced (individually) into the acceptor Ti plasmid pGV2260 by a single homologous recombination, using Sm^R gene of pJLW180 as a selectable marker for cointegration (see fig. 4 for map of cointegrate). The mobilization of pJLW180 from E. coli to Agrobacterium C58C1Rif^R (pGV2260) was performed according to Van Haute et al. (1983). The structure of the T-region was confirmed by Southern blot hybridization.

Cotyledon pieces from 12-day old aseptically germinated seedlings were inoculated with the A. tumefaciens containing pJLW180 containing either the glyphosate-resistant gene or delta-endotoxin gene (described in Section IV). After 3 days cocultivation, the tissues were placed on kanamycin media and transformed calli selected and somatic embryos were induced. The somatic embryos were germinated, and plants were regenerated and transferred to soil. Details of this procedure are described in a publication (Firoozabady et al. 1987).

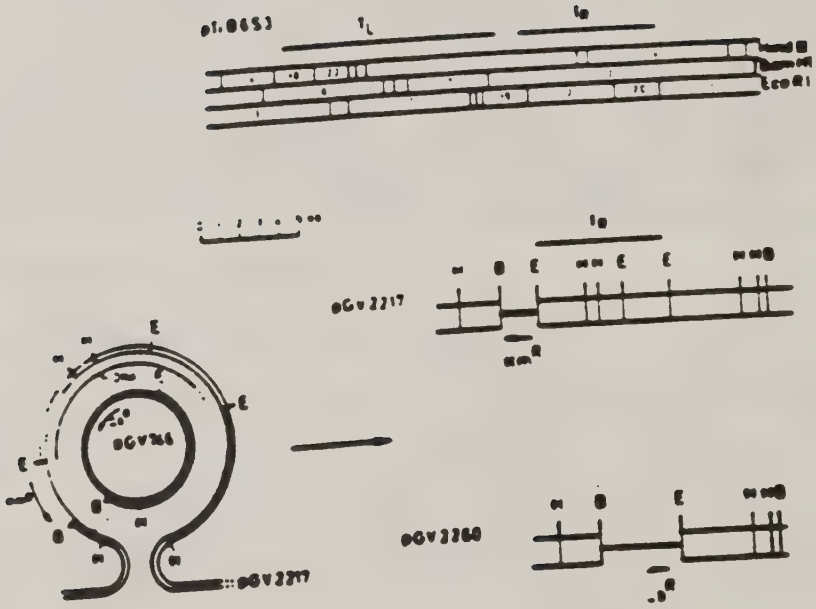


Figure 1. Construction of pGV2260.
Restriction map of the T-region of pTiB6S3 and pGV2217. In pGV2217 [6], the TL-region is substituted by a Km^R marker. The intermediate vector pGV746 was constructed as follows : the 2.3-kb HindIII/BamHI fragment from pTiAcnS fragment HindIII-14 ([///]) was cloned into pBR322, digested with HindIII and BamHI. This fragment is directly adjacent to the $left_S$ of the TL-region. The resulting plasmid, pGV713, was selected as a Cb^R Tc^S clone The pTi-region adjacent to the right of the TR-region was cloned as a 4.2-kb EcoRI/HindIII fragment, derived from pTiAcnS fragment HindIII-4 ([ ]) into pGV713 digested with EcoRI/HindIII. The resulting intermediate vector is pGV746. Recombinants between pGV746 and pGV2217 were isolated as Cb^R transconjugants after mobilizing pGV746 into CS8C1Rif^R (pGV2217) using the technique described [25]. The double cross-over events between pGV746 and pGV2217, indicated by crossed lines, were obtained by screening the Cb^R transconjugants for the loss of the Km^R marker present on pGV2217. The physical structure of one Rif^R, Cb^R and Km^S transconjugant, pGV2260, was verified by Southern hybridization and is depicted in the figure.

Figure 1. Construction of pGC2260 (the disarmed Ti plasmid)

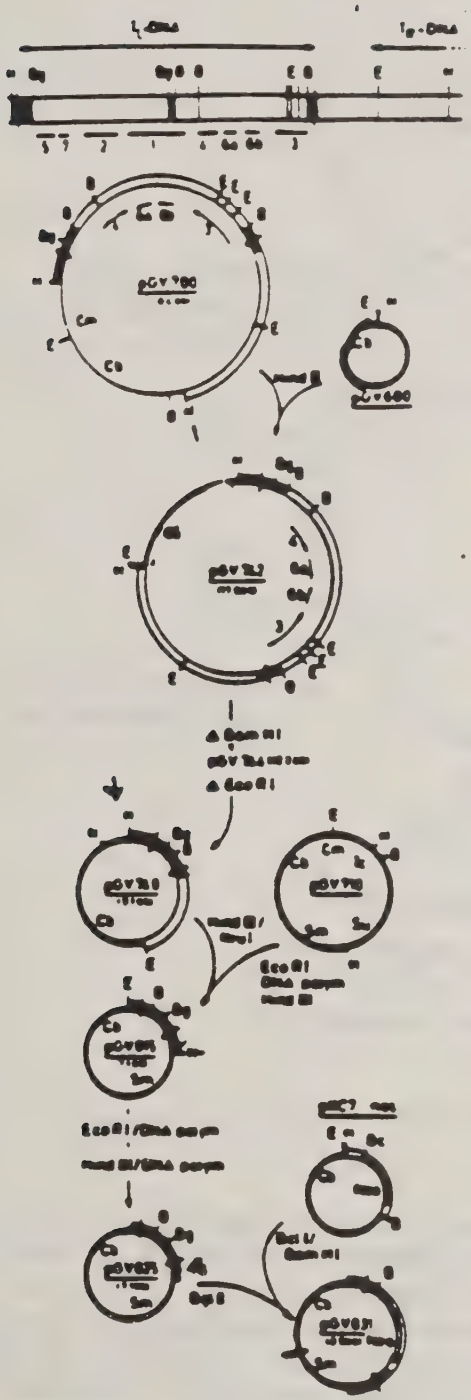


Figure 2. Construction of pGV831. The T-region of pTiB653 is presented on top of the figure. The dark fragments are those which are maintained in pGV831. The 7.5-kb HindIII fragment from pGV700 (Table 1) was recloned into pGV600, a pBR322 derivative lacking the BamHI site. The remaining TL-DNA genes in pGV742 were removed by deleting the internal BamHI fragments (pGV744). The left part of the TR-DNA was removed by deleting the internal EcoRI fragments (pGV749). pGV710 is a pBR325 derivative that contains an additional $Sm^R Su^R$ marker. To obtain pGV710 the 2.43-kb HindIII/PstI fragment from pBR325, containing the Cm^R gene, was cloned in a HindIII/PstI-digested cosmid pHC79 and the 1.62-kb BglII "cos" fragment of the resulting plasmid was substituted by a 3.45-kb BamHI fragment from the P-type plasmid R702 that encodes resistance to Sm/Sp and Su [31]. In order to obtain a fragment containing only the TL-border sequences, the 1.87-kb HindIII/ NruI fragment from pGV749 was cloned into pGV710 digested with EcoRI, and HindIII. The sticky ends obtained after EcoRI digest were flush-ended by treatment with Klenow DNA polymerase before HindIII digestion. pGV815 was isolated as a Sm^R, Cb^S, Cm^R and Tc^S clone. In pGV825 the EcoRI and HindIII sites were eliminated by filling-in the sticky ends and self ligation of the vector. A 290-bp BclI/ BamHI fragment from pLGV2381 [14] comprising the nopaline synthase promoter and cloned into the BclI site of pHC7 produced pHC7::nos. The nos promoter directs transcription of the neo gene in plant cells [14]. This chimeric Km^R gene was isolated as a BclI/BamHi fragment and cloned into the BglII site of pGV825 to produce pGV831. Abbreviations : B, BamHI; Bc, BclI, Bg, BglII, E, EcoRI; H, HindIII; N, NruI; Cb, carbenicillin; Cm, chloramphenicol; Sm, streptomycin; Sp, spectinomycin; Su, sulfathiazol; Tc, tetracycline.

Figure 2. Construction of pGV831.

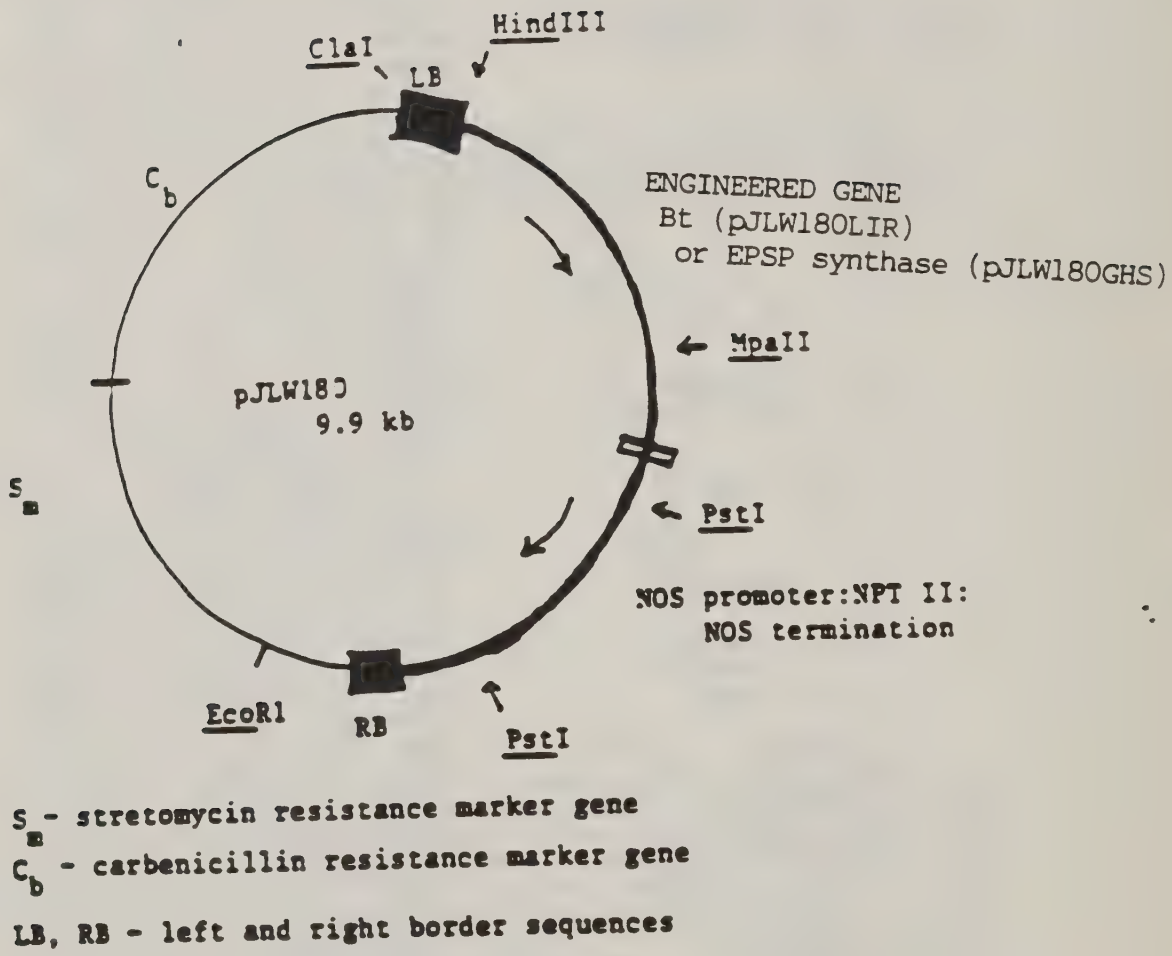
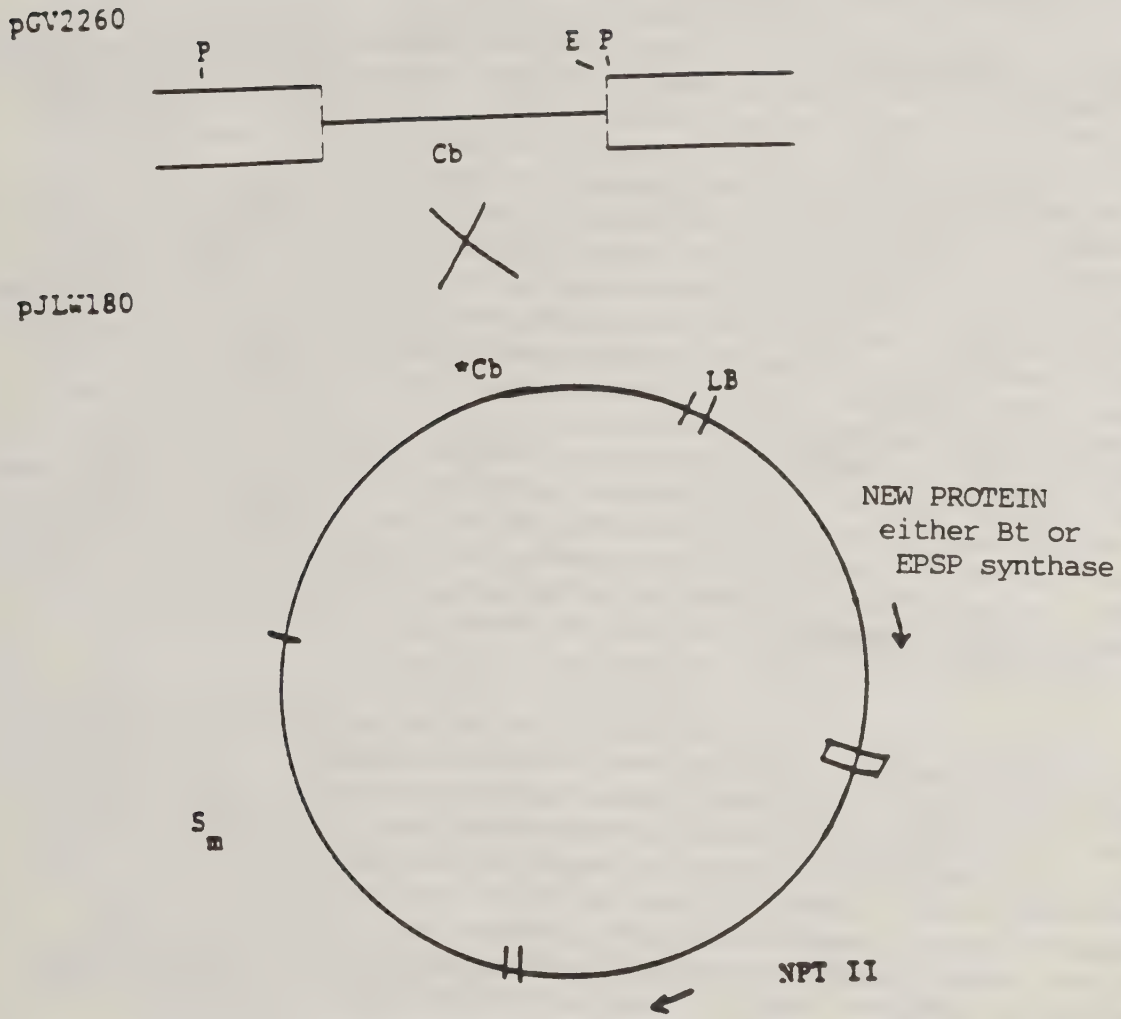


Figure 3. Map of pJLW180. The border regions are derived from an octopine type plasmid (Thomashow et al. 1980); the LB (1050 bp) and RB (550 bp). The chimeric protein contains the NOS termination and polyadenylation signal sequences (nucleotides 19,995 to 20,543 (Barker et al. 1983)) and the chimeric NPT II gene contains the NOS promoter (BamH1 fragment of pLGV2381 (Herrera-Estrella et al. 1983)). NPT II was isolated from Tn5 (Deblaere et al. 1985). S_m - streptomycin resistance marker gene; C_b- carbenicillin resistance marker gene; LB and RB -left and right border sequences.



Map of Cointegrate pJLW180::pGV2260

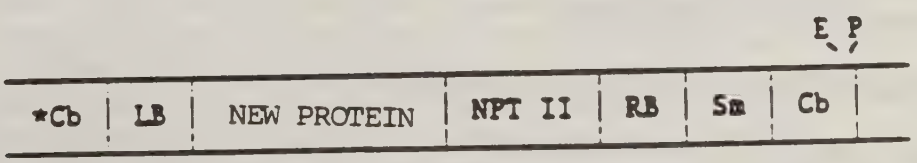


Figure 4. pJLW180::pGV2260: Map of cointegrate.

IV. The Donor Genes to be Exempted

Bacillus thuringiensis delta-endotoxin

Bacillus thuringiensis (Bt) is a rod shaped bacterium that is found in soil in most regions of the world. Different strains of B. thuringiensis exhibit a unique insecticidal activity when eaten by susceptible larvae of insect from the orders Lepidoptera, Coleoptera, or Diptera. B. thuringiensis upon sporulation produces a small crystalline protein which upon ingestion by susceptible plant-feeding caterpillars results in their death. Susceptible insects have the combination of pH, enzyme and toxin receptors in their digestive systems necessary to digest the protein and thus release the active toxic moiety. The toxin acts on the lining of the insects midgut. The osmotic balance is disrupted and the cell dies (Hofte and Whitely 1989). Action of the toxin may be so severe as to result in death or causes enough damage to stop feeding. Usually the insect is killed within 4 days. Since it is a host specific pathogen, Bt does not have many of the disadvantages associated with chemical insecticides.

B. thuringiensis HD-73 delta-endotoxin gene (Adang et al. 1987) was used to engineer the cotton plants. A truncated version of this gene was inserted into the cotton genome. This truncated gene encodes amino acids 1 through 625 of the full length protein. The sequence of HD-73 was previously published (Adang et al. 1987). The Bt gene sequence was linked to the boll-specific promoter and the NOS polyadenylation/termination sequences as previously described (White 1991). Confirmation that this gene was indeed inserted into the cotton chromosome was demonstrated by following: Southern hybridization data, Mendelian inheritance, measurement of expression levels of mRNA of Bt gene, and lepidopteran insect bioassay (see subsequent sections).

5-Enolpyruvyl-3-Phosphoshikimate Synthase Gene

The donor organism used to supply the glyphosate tolerance gene, aroA, was the bacterium Salmonella typhimurium. S. typhimurium is a well-characterized enteric bacterium with homology to E. coli (Ochman and Wilson 1987). Some strains of S. typhimurium are known to cause a disease in susceptible mice and humans, but there is no evidence that strains of the bacterium are plant pests (Le Minor 1981). Salmonella species may be associated with vegetation as free living organisms whenever these plants have been

contaminated with fertilizers of fecal origin or when watered with polluted water. Salmonella organisms do not seem to multiply significantly in the natural environment (outside of digestive tracts), but they can survive several weeks in water and several years in soil if the conditions of temperature, humidity, and pH are favorable (Delage 1960).

The aroA gene, which encodes the sequence for the enzyme EPSP synthase, has no known inherent plant pest characteristics. EPSP synthase (EC 2.5.1.19) is one of the enzymes in the biosynthetic pathway leading to chorismate, an intermediate in the formation of aromatic amino acids and their derivatives (Pittard 1987). The aroA gene is constitutively expressed in both E. coli (Tribe et al. 1976) and S. typhimurium (Gollub et al. 1983). The gene was modified in the bacterium to provide glyphosate resistance by the classical genetic techniques of mutation and selection (Comai et al. 1983). The description of the modified aroA gene, including the method of isolation and complete gene sequence, is contained in two published papers (Comai et al. 1983; Stalker et al. 1985). The EPSP synthase gene was fused to the NOS promoter and NOS termination/polyadenylation sequences as previously described (White 1991). Confirmation that this gene was indeed insert into the cotton chromosome was demonstrated by following: Mendelian inheritance, Southern data, expression levels of EPSP gene, and whole plant resistance to glyphosate application (see subsequent sections).

The Transformation Selectable Marker Gene - Neomycin Phosphotransferase

Another gene, besides the engineered protein gene, is incorporated into the chromosomal DNA after transformation. This enzyme, neomycin phosphotransferase (NPT II), confers resistance to the common aminoglycoside antibiotic, kanamycin, by phosphorylating the molecule and thereby inactivating it (Fraley et al., 1986). This gene is from transposon Tn5 and functions only as a genetic marker in the initial cell selection process following transformation. The DNA sequence of the gene has been determined (Beck et al. 1982). This enzyme and its related homologs are probably the most widely distributed of all the modifying aminoglycoside-aminocyclitol-modifying enzymes. They are found in both Gram-positive and Gram-negative bacteria (Davies and Smith 1978) including human intestinal flora. The lack of risk to

humans of the NPT II gene can be supported by its use in the first human gene therapy trials (Anonymous 1990).

The NPT II gene from pGV831 is expressed in plants although a specific polyadenylation site has not been added to the 3' termini of the gene. Presumably, the AT (adenine:thymidine) rich region at the 3' end of the gene provides the necessary sequences for termination and polyadenylation (Deblaere et al. 1985). Neither the marker gene nor the enzyme it encodes has any inherent plant pest characteristics. Confirmation of incorporation of this gene into the engineered cultivar was demonstrated by Southern hybridization data and resistance of the cultivar to kanamycin (see subsequent sections).

V. Genetic Analysis and Agronomic Performance of the Transformed Cultivar

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The data presented in this section will be divided into two section, the first dealing with general agronomic traits of the transformed herbicide-tolerant cotton and the latter containing the data unique for Bt-resistant cultivar. Applications will have to provide the comprehensive data package for each transformed plant.
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Mendelian Inheritance

The primary transformants that expressed the NPT II marker gene and the EPSP synthase gene were allowed to self-pollinate and the seeds were collected. These seeds (T_1) were planted in a single 25 foot row. Seedlings were thinned to a density of two plants per foot. Seedlings were sprayed with one application of glyphosate at a rate of 8 oz./acre. Symptoms of bleaching or necrosis appeared 8 to 10 days after application. Symptoms were compared to nontransformed plants that received an identical herbicide application. The number of resistant and sensitive plants in three separate rows were counted (table 1). If primary transformants had a single expressed copy of the EPSP gene the expected segregation would be 3:1 (37½:12½ for 50 plants per row). The segregation ratio totals for all 3 rows was 110:40 versus the expected ratio 112½:37½ ($\underline{X}^2$=0.22). The nontransformed plants were fully susceptible.

Table 1. Segregation ratios of progeny of the seeds of the primary transformants.

	Ratio of Resistant plants to Susceptible plants		
	Observed	Expected	$\underline{X}^2$
Stoneville 825 GHS	36:14	37½:12½	0.24
Stoneville 825 GHS	39:11	37½:12½	0.24
Stoneville 825 GHS	35:15	37½:12½	0.67
Stoneville 825	0:50	0:50	--

The resistant T_1 plants were grown to maturity. Flowers were bagged prior to pollen shed to ensure self-pollination. Seed (T_2) from each tolerant T_1 plant was collected and bulked. To confirm that a single gene was

present, pollen from T_1 plants was used to pollinate emasculated nontransformed Stoneville 825 cultivar. Seed was allowed to mature and individual bolls were harvested. Seeds were planted in the field as described above and treated with herbicide. The ratio of herbicide resistant to sensitive plants was 70:30 (X^2= 0.41). The expected ratio for a single gene in a backcross would be 0.67:0.33.

Southern Data and Copy Number

To determine if more than one of the glyphosate-resistant EPSP synthase and one copy of NPT II marker gene were present in the engineered cultivar, Southern analysis of genomic DNA was performed. Southern analysis was performed as previously described (Firoozabady et al. 1987) and the results shown in figure 5 for EPSP synthase gene and figure 6 for NPT II marker gene. The data supports the Mendelian results that only one expressed copy of the glyphosate-resistant EPSP synthase gene is present in the engineered cultivar and that a single copy of the NPT II marker gene is present.

In summary, the Mendelian and Southern analysis support the hypothesis that a single copy of the engineered genes have been incorporated into the chromosomal DNA.

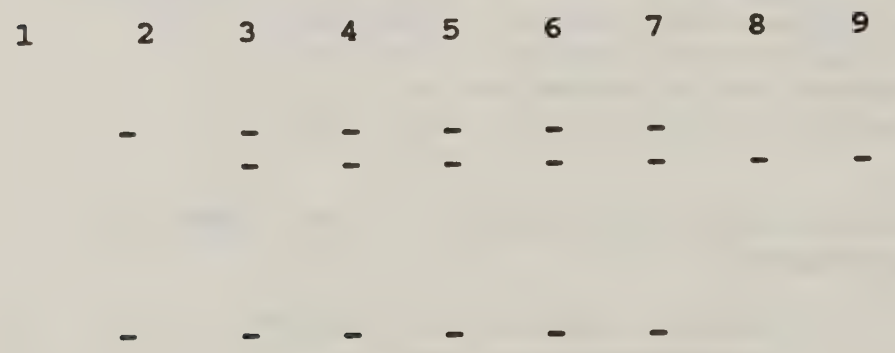

Figure 5. Detection of DNA sequences in the transformed cotton plants. Genomic DNA was isolated from nontransformed plants (lane 1), transgenic plant containing the vector alone (lane 2), and five F_5 transformed plants vector plus the engineered gene (lanes 3 to 7). DNA was digested with BBEPII and BPUI separated on 1% agarose gels, transferred to nitrocellulose, and hybridized with ^{32}P-labeled ssRNA transcripts of the cDNA. Reconstructions represent one copy and 5 copies per genome equivalent of the engineered gene (lanes 8 and 9, respectively).

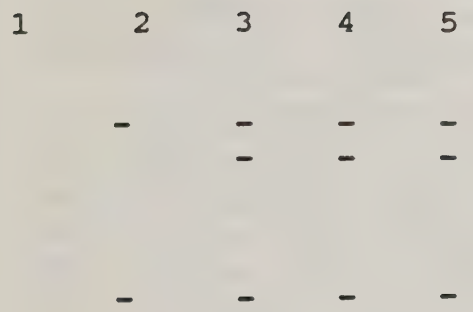

Figure 6. Identification of NPT II (kanamycin marker gene) DNA sequences in the engineered cotton plants. Genomic DNA was isolated from nontransformed plants (lane 1), transgenic plant containing the vector alone (lane 2), and five F_5 transformed plants vector plus the transformed gene (lanes 3 to 5). DNA was digested with BBEPII and BPUI separated on 1% agarose gels, transferred to nitrocellulose, and hybridized with ^{32}P-labeled ssRNA transcripts of the cDNA to NPT II. Reconstructions represent one copy per genome equivalent of the engineered gene and shown in lane 6.

Disease and Pest Characteristics of the Transformed Cultivar

The transformed cotton plants were field tested for 3 years at various sites in the mid-South region of U. S. Based on field observation at these sites the transformed cultivar pathogen susceptibility or resistance characteristics were unchanged when compared to its nontransformed cultivar. The transformed cultivar remains resistant or tolerant to bacterial blight (*Xanthomonas campestris* pv. *malvacearum*), *Anthracnose* boll rot, and *Fusarium* wilt-nematode complex rot. The transformed cultivar remains susceptible to *Alternaria* leaf spot, *Cercospora* leaf spot, and powdery mildew, as was the nontransformed cultivar. The disarmed *A. tumefaciens* strain was not isolated from transformed plants in the field and would not expected to be present since *Agrobacterium* is not seed transmitted in cotton.

Cottonseed Yield and Quality Factors

Every ton of cotton picked in the field yields about 600 kg of seed and 350 kg of cotton lint, but the value of the lint is about seven times that of the seed. Seed is therefore treated as a by-product but it can provide significant quantities of edible oil and a protein-rich food for livestock. The seed is cut or cracked and the

hulls separated mechanically from the meat. The oil can be extracted mechanically (with a screw press) or chemically with a solvent (n-hexane). The seeds (meats) are heated or cooked to enable the oil to flow freely and to rupture the oil-bearing cells. This also reduces the free gossypol in the cake or meal, as most of it is fixed by chemical combination with other constituents of the seed. Cotton seed oil has be refined to get rid of the inherent impurities, free fatty acids, color, gums and odor. Cotton seed extractions, with about 40% protein, are standard component of animal feeding stuffs (Munro 1987). An outline of the many uses of products derived from cottonseed is shown in figure 7.

LINT AND SEED YIELD. The yield of lint, both first harvest and total, were not significantly different between the transgenic and nontransgenic cultivar (table 2). The yield of seed from the transgenic versus the nontransgenic cultivar was virtually identical (table 2).

GOSSYPOL AND FLAVONOLS. Gossypol is a yellow coloring matter which occurs in various parts of the cotton plant. Cotton seed usually contains 0.4% to 1.7% gossypol (Abou-Donia 1976). Gossypol is toxic to animals when present in untreated cottonseed meal. When cottonseed meal is processed under heat and moisture, most of the free gossypol is removed by solvent extraction or detoxified by the condensation of the aldehyde groups of gossypol with the free amino groups of proteins to form non extractable (bound) gossypol. The amount of free or bound gossypol in the meal was not significantly different in the transformed as compared to the nontransformed cultivar (table 3).

One of the common plant phenolic pigments, the flavonoids, is mutagenic in the Salmonella/mammalian microsome assay (Brown, 1980). More than 2,000 individual flavonoids have been described, and as a group are universally distributed among vascular plants. Most of the flavonoids in plant materials are in the form of glycosides. For quercetin (3,3',4',5,7-penta-hydroxyflavone), the most common flavonol, over 70 glycosidic combinations have been characterized (Brown 1980). Flavonoids are important allelochemicals for normal plant growth, development, and defense against infection and injury by phytophagous pests. Flavonoids affect the behavior, development, and growth of number of insects. Flavone glycosides and aglycons in the cotton plant are also larval growth inhibitors for bollworm and budworm (Hedin et al. 1988).

No differences were found in flavanoid amounts between the transgenic and nontransgenic cultivar (table 3).

FATTY ACIDS. Cottonseed oil contains sterulic and malvalic acids - 18 and 17 carbon cyclopropenoid fatty acids, respectively, which contain one double bond at the site of a propene ring, either at the 9,10, or 8,9 positions. The cylcopropene ring is chemically and physiologically reactive. Feeding studies have shown that these changes in the ratios of these fatty acids into poultry and animal feeds causes the hardening of fats in egg yolk and milk (Johnson et al. 1967; Bickerstaffe and Johnson 1972; Roehm et al. 1970). The free fatty acid and cyclopropene fatty acids in the transformed cultivar were not significantly different (table 3).

AMINO ACID COMPOSITION OF COTTONSEED PROTEIN. Since cottonseed meal protein is an important feed supplement for livestock (figure 7), we compared the amino acid composition of meal protein from transformed and nontransformed cultivars (table 4). No significant differences in amino acid composition was found.

AMOUNT BT IN OIL AND PROCESSED COTTONSEED MEAL. Processed plant oils are protein free and thus would not contain Bt proteins. Since the promoter used to express Bt in plants is boll/square-specific, the amount of Bt expected be to present in seeds would be extremely low. Using PCR (polymerase chain reaction) with primers specific for a 250 nucleotide portion of the Bt gene, on total RNA preparations from seeds, no specific Bt-related RNA could be detected. To determine if Bt was stable during the processing of the seeds into oil and meal, purified truncated Bt (isolated from E. coli) was added after seeds (10 ng per gram seed) after they were dehulled but prior to hexane extraction and heating. At final processing, no Bt protein was detected by immunoassay.

AFLATOXINS. Aflatoxins are most commonly found in food and feed commodities contaminated by Aspergillus flavus. Aflatoxins are the only contaminants of feeds and food routinely monitored. If in the unlikely event that seeds from the transformed cotton plants are significantly different from their nontransformed counterparts in their ability to support A. flavus growth, this would be readily detected by current procedures.

Figure 7. Select industrial products from cottonseed.

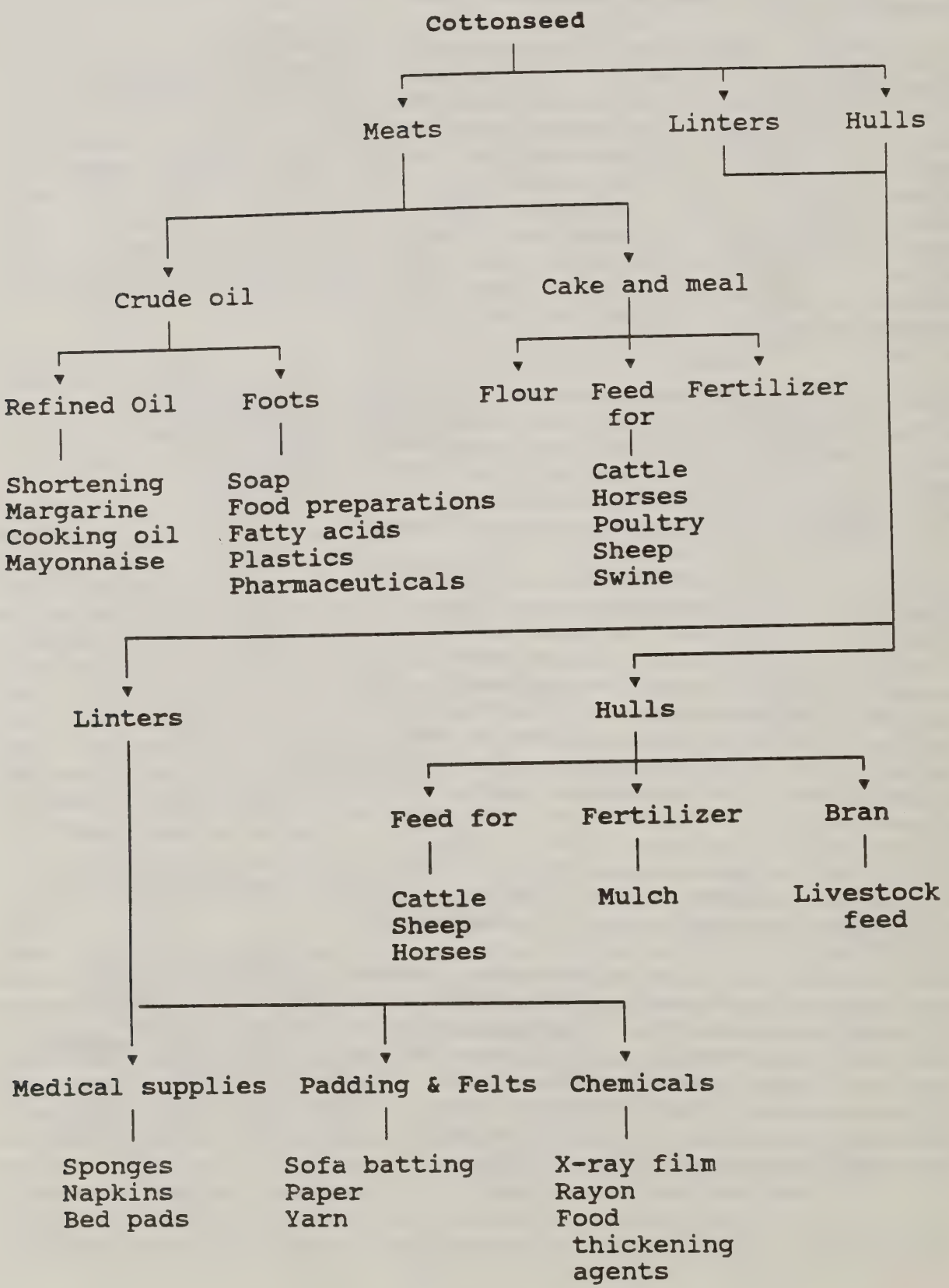

Table 2. Average yield of lint and seed from transformed and nontransformed cotton from 5 different environments.

Plant	Lint yield		Lint	Weight	
	Total	First		Boll	Seed
	kg ha^{-1}		%	mg	
Transformed	950a	500a	35.7a	500a	104a
Nontransformed	955a	499a	35.7a	497a	103a

[a]Within column means followed by the same letter are not significantly different at the 0.01 probability level as indicated by a t-test (Meredith 1990).

Table 3. Mean values of cottonseed quality traits of transformed and nontransformed cultivars grown at 4 sites.

Quality factors	Transformed	Nontransformed
Oil	19.04a	19.09a
Protein	23.44a	22.25b
Free fatty acids	0.86a	0.87a
Free gossypol	0.75a	0.75a
Total gossypol	1.0a	1.0a
Cyclopropene fatty acids	0.83a	0.82a
Flavonoids	1.84a	1.76a

[a]Means among cultivars having the same letter are not significantly different according to the Newman-Kreuls multiple range test.

[b]Oil and protein are percent of linted seed; free fatty acid is percent of oil; free, total gossypol, flavonoids are percent of kernels; and cyclopropene fatty acids as percent of total fatty acids (Cherry 1983; Hedin 1988).

Table 4. Amino acid composition of cottonseed protein (based on Zarins and Cherry, 1981).

Amino acid	Nontransformed	Transformed
	g/100 g dry weight protein	
Lysine	3.4	3.5
Histidine	2.9	2.8
Arginine	10.0	9.9
Tryptophan	-	-
Cystine	-	-
Aspartic acid	9.0	9.1
Threonine	2.9	3.0
Serine	4.0	3.9
Glutamic acid	16.4	16.3
Proline	3.4	3.3
Glycine	3.5	3.5
Alanine	3.6	3.6
Valine	4.7	4.8
Methionine	1.4	1.3
Isoleucine	3.4	3.3
leucine	5.7	5.8
Tyrosine	2.8	2.7
Phenylalanine	5.7	5.7

Pollination characteristics of the transformed cultivar

We determined the movement of pollen from a field for the transformed cultivar at our Alabama field test site. A field test of transgenic cotton measuring 150 meters by 30 meters was bordered on all sides by 25 meters of commercial cotton. All border rows were sampled from the lower, middle, and top fruiting positions of the plants. Seeds were analyzed for expression of the selectable marker NPT II by germinating seeds on agar containing kanamycin. Potential transformants were confirmed by PCR analysis. Results (figure 8) showed a consistent reduction of pollen dissemination as distance from the test plot increased. Outcrossing declined from 6% to less than 1% by 10 meters away from the test plot. A low level of pollen dispersal of less than 1% continued to occur sporadically in the remaining border rows out to a distance of 30 meters. No significant differences were noted for flower position on the plants and the occurrence of NPT II-expressing seeds, indicating there was no consistent seasonal effect on pollen distribution.

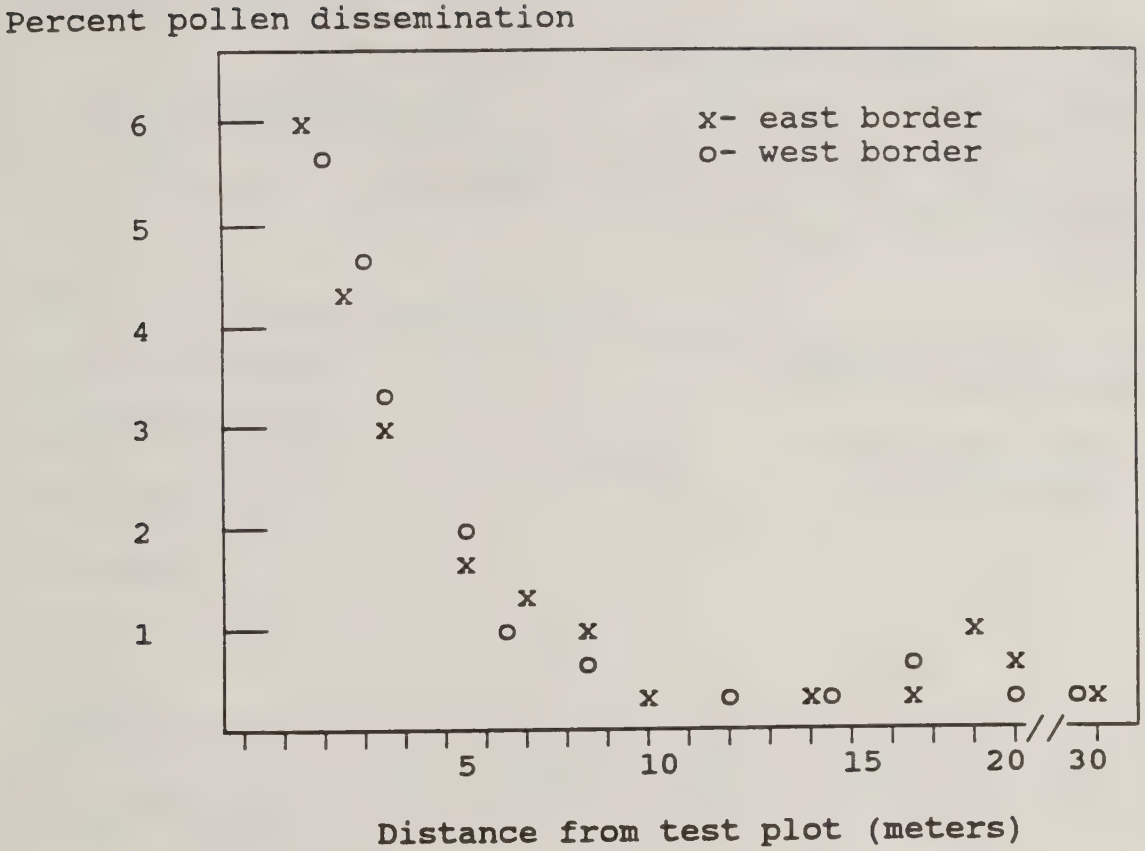

Figure 8. Movement of pollen from transformed plants to border plants as determined by presence of marker gene in progeny plants from border plants.

Characteristic of Glyphosate-tolerant cotton

We determined the minimum level of glyphosate needed to be applied to control the major cotton weed pest, morningglories, and not cause damage to cotton plants (table 5). The minimum level of glyphosate that could be applied which caused no damage to cotton and effectively control moringglories was 8 ounces per acre.

To compare the effectiveness of glyphosate versus the other commonly used cotton herbicides, a field trial was planted with the transformed and nontransformed cultivar and subsequently a mixture of weed seeds was spread over the whole field. Herbicides were applied when either the cotton and the weeds were at appropriate size or herbicide application. As shown in table 6 glyphosate was effective in controlling all the most common weeds in cotton

producing areas and was superior to other commonly used herbicides.

The glyphosate-tolerant cotton is still susceptible to two other broad spectrum herbicides, sulfonylurea and bromoxynil, as is its progenitor cultivar (table 7). Thus, if the transformed cultivar is not resistant to all herbicides and if the transformed cultivar needed to be eliminated for some unforeseen circumstances, several herbicides are available.

Table 5. Level of application of glyphosate and moringglory weed control.

Glyphosate application level (oz./acre)	Leaf damage rating[1]	Weed Control rating[2]
0	0	0
2	0	3
4	0	4
6	0	6
8	0	8
10	1	9
15	2	9

[1] Leaf damage rating: 0- no damage; 1-slight chlorosis/necrosis; 2- visible damage includes chlorosis and necrosis; 3- severe leaf damage; 4- plant defoliation within 2 weeks after application.

[2] Weed control rating scale: 0-3, none to slight; 4-6, fair; 7-8, good; 9-10, excellent.

Table 6. A comparison level of weed control by application of commercially registered cotton herbicides on nontransformed cultivar versus glyphosate on the transformed cultivar.

	Herbicides			
Weeds	Glyphosate	Fluazifop	Fluometuron and MSMA	DMSA or MSMA
Morningglory	8	0	8	3
Common cocklebur	9	0	9	9
Pigweed	9	0	9	3
Johnsongrass	7	9	5-8	5-8
Nutsedges	8	0	6	6
Bermudagrass	7	9	0	0

[1] Weed control rating scale: 0-3, none to slight; 4-6, fair; 7-8, good; 9-10, excellent. Herbicides were applied in the manner suggested in the guidelines and according to the label under optimum growing conditions.

Table 7. Susceptibility of glyphosate tolerant cotton to bromoxynil and sulfonylurea herbicides.

	Leaf damage rating[1]	
	Nontransformed	Glyphosate-tolerant
No herbicide	0	0
Sulfonylurea		
1/6 oz./acre	2	1
1/3 oz./acre	3	3
1/2 oz./acre	4	4
Bromoxynil		
1/2 pt./acre	1	1
1 pt./acre	2	2
1 1/2 pt./acre	3	4
Glyphosate		
8 oz./acre	4	0

[1]Leaf damage rating: 0- no damage; 1-slight chlorosis/necrosis; 2- visible damage includes chlorosis and necrosis; 3- severe leaf damage; 4- plant defoliation within 2 weeks after application.

Expression Levels of Bt in Plant Tissues and Products

INSECT BIOASSAY. Insects (M. sexta, T. ni, H. virescens, H. zea, S. exigua) were placed directly on young bolls of wild-type or recombinant plants, usually 8 larvae per plant per test with up to six successive tests conducted per plants. At the end of the assay the larvae were scored for mortality and weight gain and the plants were rated for amount of damage (Barton et al. 1987; Fischoff et al. 1987). These results are shown in table 8. Alfalfa weevil, cotton bollweevil, Southern corn root worm, mosquito, cockroach, green peach aphid termite, and spider mite were also tested and found insensitive to B. thuringiensis HD-73. The relative sensitivities or insensitivity of these insects to Bt HD-73 are similar but not identical to those reported for the toxicity of cloned HD-73 Bt (but not expressed in plants) (MacIntosh et al. 1990). This data is reproduced in table 9. The differences may be due the variation in feeding behavior on plants (table 8) versus artificial diet used in the experiments described in table 9.

Table 8. Relative sensitivity of lepidopteran insects to Bt HD-73 expressed in cotton bolls and squares.

Insect	HD-73 LC_{50}[a]
Heliothis zea	0.01
Manduca sexta	0.2
Heliothis virescens	0.4
Trichoplusia ni	10
Spodoptera exigua	22
Ostrinia nubilalis	99

[a] LC_{50} is the concentration of Bt protein causing 50% mortality.

Table 9. Relative sensitivity of target lepidopteran insects to cloned Bt HD-73 (data reproduced from MacIntosh et al. 1990).

Insect	HD-73 LC_{50}[a]
Manduca sexta	0.036
Trichoplusia ni	0.09
Heliothis virescens	1
Heliothis zea	20
Spodoptera exigua	44

[a]LC_{50} is the concentration of Bt protein causing 50% mortality.

QUANTITY OF BT EXPRESSED IN DIFFERENT PLANT TISSUES. We have attempted to quantify the amount of Bt produced in various plant tissues by two methods: Northern hybridizations and insect bioassays. Northern analysis for mRNA was chosen over protein detection methods for several reasons: (1) because the Bt protein is proteolytically cleaved into smaller polypeptides when synthesized in plants, immunological methods may present problems in determining total amount present; (2) because different plant tissues may have different proteinase enzymes at different concentrations thus complicating comparisons between different tissues; and (3) because the promoter for Bt gene was boll-specific, the levels of expression in some tissues were expected to be very low. Northern analysis of mRNA from boll tissues (bract and corrolla), root,leaf mesophyll, and stems revealed Bt-specific mRNA only in the boll specific tissues (see figure 9). Dot blot analysis revealed that amount of Bt specific RNA in bract/square tissue was approximately 0.01 ng per mg of total RNA (figure 9).

As an additional approach to Bt expression in plants, tobacco budworm (H. virescens), bollworm (H. zea), and cotton leafworm (S. exigua) were "caged" on either boll/square tissues or leaf/stem tissues from either the transformed or nontransformed plant. Only the two lepidopteran insects of the bollworm complex were killed when they fed on boll/square tissues from the transformed cultivar. The nontarget cotton leafworm pest viability was not significantly affected by synthesis of Bt in boll/square tissues (table 8).

Figure 9. (a) Northern hybridization analysis of Bt mRNA in different cotton tissue parts. RNA was isolated as previously described (White 1991) and the RNA fractionated on formaldehyde-containing gels and blotted to nitrocellulose membrane. The membrane was hybridized with ^{32}P-labeled BamH1-PstI fragment from plasmid pJLW180bt containing the nearly 90% of Bt gene. Sources of tissue analyzed: lane 1- bracts/corrolla; lane 2- root; lane 3 - leaf mesophyll; lane 4 - stem; and lane 5- mRNA transcript from cloned Bt DNA. (B) Estimate of Bt mRNA levels in tissue. Known quantities of Bt mRNA or total RNA from bracts/squares were blotted to nitrocellulose membranes. The membrane was treated as described above.

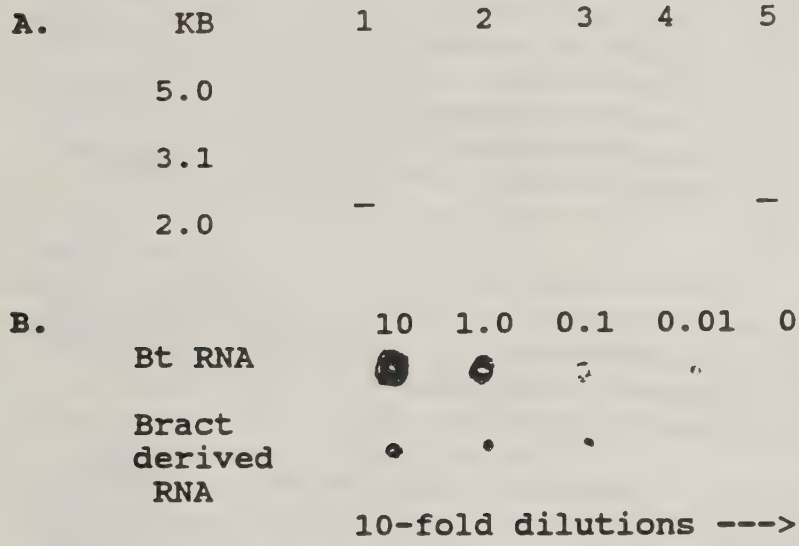

Table 10. Toxicity of select plant tissues to lepidopteran larvae.

Tissue type	Plant type	Mortality[1] (in percent)		
		H. virescens	H. zea	S. exigua
Boll/ squares	Transformed	100	100	10
Leaf	Transformed	20	10	0
Boll/ squares	Nontransformed	0	0	0
Leaf	Nontransformed	0	0	0

[1] Insect bioassays performed as described above and in Fischoff et al. 1987.

Comparison of Lepidopteran Insect Control and Numbers of Beneficial Insects on Bt-expressing Cultivar versus Nontransformed Cultivar under Field Conditions

A comparison of the effectiveness of the Bt-expressing plants in controlling the bollworm complex insects is shown in table 8. The percent squares and bolls injured in the Bt-expressing cultivar were slightly higher when compared to the nontransformed cultivar that was treated with pyrethroids. However, seed yield from the two plots was not significantly different. The number of predatory insects (pirate bugs (*Osrius* spp.), big-eyed bugs (*Geocoris* spp.), green lacewings (*Chrysopa* spp.), chalid fly (*Trichogramma minutum*) was significantly higher in the plots of the transformed cultivar than the insecticide-treated cultivar. These predatory insects besides feeding on bollworm complex insects also feed on other cotton insect pests. thus other insects whose populations were not determined were also probably reduced when the transformed cultivar was compared to pyrethroid-treated nontransformed cultivar.

Table 11. Effectiveness of cotton cultivar expressing Bt versus chemical insecticides control on the nontransformed cultivar against tobacco budworm and bollworm in field tests and numbers of predatory insects.

Treatment	Percent squares injured	Percent bolls injured	Seeds/ acre (lb.)	Number predators/ five plants
No insecticide on cultivar	74.2	82.5	170	20
Pyrethroids on cultivar	5.2	6.4	940	3
Bt-expressing cultivar	9.3	7.4	929	25

All plots sprayed at recommended intervals based on IPM.

VI. Environmental Consequences of Introduction of the Transformed Cultivars

The Herbicide Glyphosate

N-(phosphonomethyl)glycerine (glyphosate) is an extremely effective broad spectrum herbicide. The primary mode of action of the herbicide appears to be competitive inhibition of 5-enolpyruvylshikimic acid-3-phosphate (ESPS) synthase, an enzyme in the shikimic acid pathway of aromatic amino acid biosynthesis. Glyphosate provides effective control for the majority of the world's worst weeds. It is translocated in the plant via both phloem and xylem. The broad spectrum herbicidal activity is only evident when glyphosate is applied to foliage, as there is little penetration of bark or woody stems (Franz 1983). Glyphosate is nontoxic upon contacting soil. Its degradation appears to be mainly microbial. Glyphosate is essentially nontoxic to mammals and birds (Anonymous, 1983). Environmental impact studies indicate that the herbicide has little direct effect on animal communities (Sullivan and Sullivan 1979, 1981, 1982). However, some bird communities may show decreased population densities due to destruction of habitat caused by herbicidal use (Morrison and Meslow 1984). Fish and invertebrates are more sensitive to the herbicide, especially to the commercial formulations, as a result of the surfactants in the formulation (Anonymous 1983). Effects of the herbicide on soil invertebrates in field situations appear to be minor (Eijsackers 1985). Although there are numerous reports on effect of glyphosate on microbial respiration, nitrogen cycling, and cellulolytic activity in soils, no toxicity to any of these microbial processes should be observed at recommended field application rate of the herbicide (Carlisle and Trevors 1988). No reported problems have been associated with the use of glyphosate and groundwater contamination (Goldburg et al. 1990).

Current uses of Glyphosate and other Herbicides on Cotton

Glyphosate is generally used as a foliar-applied herbicide. It is most effective for the control of perennial weeds. It is usually applied before planting to kill winter weeds growing on beds listed in fall or winter or used as a spot spray at any time through out the growing season. Glyphosate is also used for destroying weeds outside the field.

Herbicides are applied to cotton (preplant foliage or soil incorporated applications), at planting (preemergence

applications), or after seedlings emerge (postemergence directed or over-the-top). Herbicides were used on 99% of the cotton acreage (2.7 million acres) in the delta region of the U.S. in 1990. In this region, cotton producers applied from 3.6 to 4.1 treatments per acre. The severe weed pressure in the Delta is demonstrated by the large proportion of the cotton acreage receiving 3 or more herbicide treatments per season. At least one-quarter of the acreage in the Delta receives application of arsenic based herbicides (DMSA or MSMA), singly or in combination with other herbicides. The total amount of arsenic based herbicides applied to cotton in 1990 was approximately 3.5 million pounds.

The management of weeds by cotton producers in the U. S. is unique for the various production regions. The following summarizes typical practices for the mid-South region of the U. S. (Frans and Chandler 1989).

1. Disk twice and broadcast and incorporate trifluralin before planting.
2. At planting apply fluometuron preemergence on bands.
3. Cultivate and postdirect fluometuron plus MSMA on bands.
4. Cultivate and postdirect prometryn plus MSMA on bands.
5. Spot spray with fluazifop.
6. Cultivate and postdirect cyanazine on bands.
7. Hand hoe, cultivate and postdirect dinoseb on bands.

Several postemergent herbicides are registered for use in cotton. These herbicides are usually applied when the plants are 3 to 6 inches high. These herbicides include diuron, fluometuron, prometryn, cyanazine, plus MSMA or DMSA to broaden spectrum. One additional application of the mixture can be made during the season since there is limit of two applications of the arsenical herbicides. Late postemergence herbicides are sometimes applied at or near the time of the last cultivation ("layby"). Direct application are usually placed between the rows, with the objective of maintaining cotton seed quality. There are a few over-the-top herbicides available. The two used are sethoxydim and fluazifop. Both are specific for control of grass weeds and have little effect on broadleaf plants. Prior to sethoxydim and fluazifop introduction, glyphosate was used to control grasses. Because glyphosate is nonselective, unique application methods were devised.

Recently cotton lint yield has declined, and continued herbicide use is strongly implicated, especially where cotton is grown continuously and the same herbicides

applied yearly (Frans and Chandler 1989). Rogers et al. (1985, 1986) summarized results from long-term experiment in which herbicides were applied to cotton at different levels for 6 to 7 years. No cotton yield reduction occurred following continuous use of a minimum set of herbicide practices. When intensive practices were used (trifluralin preplant incorporated, fluometuron preemergence, two postemergence directed applications of fluometuron plus MSMA, and linuron applied at last cultivation), yield reductions over-all averaged up to 8 percent. Of the rotation crops planted on these areas, corn and sorghum suffered the least damage while soybeans and rice were severely injured.

Herbicide residues in the cotton crop have also been a concern especially of organic arsenicals. Both DMSA and MSMA are used postemergence for control of grasses. Although most producers apply arsenicals in a directed manner, some apply them over-the-top. In the latter case, there is the possibility of high residue levels occurring in cotton, especially if applications are made during the early reproductive stage of cotton growth and if there are multiple applications (Frans and Chandler, 1989).

Glyphosate-tolerant Cotton

The introduction of glyphosate-tolerant cotton will be environmentally compatible for the following reasons:

(1) It offers producers the option of replacing with glyphosate several herbicide combinations that include arsenical compounds,
(2) Glyphosate is a herbicide which is less likely to lead to the development resistant weeds (Benbrook 1991),
(3) The introduction of glyphosate-tolerant cotton is compatible with IPM. Producers would have the option of applying a herbicide only if there was a weed problem thus reducing the use of premergent herbicides,
(4) Glyphosate is one of the most environmentally-compatible herbicides whose most damaging components are its "inert" components (Goldburg et al. 1990),
(5) Glyphosate does not have carry-over problems, and
(6) The development of glyphosate-tolerant cotton could aid in the development of minimum till practices which is not possible currently and which would result in reduced soil erosion.

Glyphosate-tolerant cotton could provide producers the option of using the herbicide on an as-needed basis, a key principle of all IPM systems. If a farmer planted a field

with herbicide-tolerant variety, the farmer could cut back initial herbicide application or try to control weeds with mechanical cultivation. If chemical weed control becomes necessary, herbicide could be applied over-the-top to the entire field or by spot application in the areas of field where weeds are threatening.

The Likelihood of the Appearance of Glyphosate-resistant Weeds

A decade ago, herbicide resistant weeds were virtually unknown. Today there are some 109 herbicide-resistant weed biotypes with over half of them resistant to triazine (Le Baron 1991). Major factors critical for the development of resistant weeds include: a single target site and a specific mode of action, broad spectrum of activity, long residual activity and the capacity to control weeds year-long, and frequent applications without rotation to other herbicides or cultural control practices. Using these criteria and based on current uses data, glyphosate is considered to be a herbicide with low risk for weed resistance (Benbrook 1991).

Major Lepidopteran Insect Pest of Cotton

The major lepidopteran pests of cotton are the bollworm (Heliothis zea) and the tobacco budworm (Heliothis virescens). The bollworm and tobacco budworm are similar in their biology so they will be discussed together. Bollworms and budworms cause significant losses by feeding on green bolls. Older larvae do the most damage, but control measures must be aimed at small larvae because large ones are hard to kill. Losses due to Heliothis are greatest where natural enemies have been destroyed by insecticides applied for other pests. The most important natural enemies of Heliothis are such predators as bigeyed bugs, minute pirate bugs, damsel bugs, and lacewings. They feed on eggs and small larvae, killing them before they reach the more damaging later instars. Bollworms and budworms feed on a variety of crops, ornamental, and weeds. In certain areas of the country, cotton is not the strongly preferred host. Corn, tomatoes, soybeans, and other crops are the preferred hosts and are often attacked first. Later generations move to cotton after the other crops have matured (Anonymous 1984)

Control of budworms and bollworms is complicated by their tolerance or resistance to many common insecticides, including organophosphates and carbamates. Larvae of either species are difficult to control with any material

currently available once they reach the third instar. There is evidence that tobacco budworm is developing significant resistance to the widely used synthetic pyrethroids. Development of a high level of resistance to pyrethroids will greatly limit the choice of chemical controls. Pyrethroids are efficacious but when used indiscriminately can cause major disruptions of secondary pests, such as spider mites (Sterling et al., 1989).

Microbial insecticides such as B. thuringiensis (as spores) and baculoviruses (Elcar®) have been used successfully in some cases, but the degree of control has been too variable for these materials to be relied upon as the sole means of control in commercial cotton fields (Munro, 1987).

No endangered or threatened lepidopteran insects that could potentially be affected by the introduction of Bt-resistant cotton plants are listed in 50 CFR 17.11 and 17.12. Currently, commercial formulations of Bt are EPA-registered to be applied to cotton.

The Effect of Bt-expressing Cotton on Predatory Insects

A significant advantage of using plants that express pesticidal products instead of conventional pesticides is that the pesticidal plants are unlikely to harm natural enemies. Pirate bugs (Orius spp.), big-eyed bugs (Geocoris spp.), green lacewings (Chrysopa spp.), chalid fly (Trichogramma minutum) are the major predators of Heliothis spp. These beneficial insects are often adversely affected by traditional chemical insecticide applications (Sterling et al. 1989). Field and laboratory tests of conventional Bt formulations on cotton (ref) have demonstrated that these insects are not significantly affected by the insecticide. We have observed similar results during the field testing of the transformed cultivar expressing Bt. These results are not unexpected since this Bt's host range is limited to lepidopteran insects.

Development of Insects Resistant to Bt

Within the last five years, the capacity for high levels of insect resistance to Bt has been reported in several important insect pests including: tobacco budworm, Colorado potato beetle (Leptinotarsa decemlineata), Indianmeal moth (Plodia interpunctella), almond moth (Cadra cautella), and diamondback moth (Plutella xylostella). Thus far, significant resistance has

occurred in the field only in the diamondback moth. With gypsy moth (Lymantria dispar), increased dosages have been required in aerial spraying programs over the past few years although this has not been directly attributed to resistance (McGaughey 1991).

Population models (Gould 1988a,b,c) indicate that under certain conditions it may take longer to adapt to a toxin if it is only produced in specific plant parts that are most sensitive to insect feeding. Experiments conducted in the laboratory (Gould et al. in press) have shown that expression of Bt genes in tobacco buds but not in older leaves cause H. virescens to avoid sensitive bud tissue. These are important attributes to consider when assessing integrating these plants into an IPM system.

JPS Biotechnologies expect that Bt-resistant Heliothis spp. will develop if these resistant plants are widely planted in the Delta region. Gould (1988 a,b) has proposed several approaches to delay appearance of resistant biotypes: (1) expression of insecticide in select tissues; (2) planting of seed mixtures that contain 80% expressing- and 20% nonexpressing cultivar; (3) expression of two insecticidal products in the plant, each of which has a different target site.

We have taken the approach of expressing the Bt gene only in the critical tissue on which the insects feed, the bolls. The percent of squares and bolls injured and the yield of seeds were nearly identical when comparing the Bt-resistant cultivar (table 11) and pyrethroid-treated nontransgenic cultivar. An additional advantage of using the transformed cultivar is that the numbers of predatory insects on the transformed plants was significantly higher than on the insecticide treated plants.

The feeding practices of insects of the bollworm complex will slow the development Bt- resistance. Heliothis spp. can breed on a very wide range of plant species, including many cultivated crops like: corn, sorghum, tobacco, and cowpeas (Munru, 1987). These alternative plants allow the bollworm to maintain or increase its numbers prior to attacking cotton or may divert the attack from cotton, depending on the relative acreage, timing and attraction of cotton crop and the host plants. With respect to the insects feeding on Bt-expressing cotton, these other plants offer an environment, which portions of the populations can develop free of any selection pressure, thus assuring dilution of any selection that may be occurring on the transformed crop plants (McGaughey 1990).

As with any pesticide is prudent that Bt whether applied externally or *in planta* be used in a manner that will prolong its effectiveness.

Weediness of the Transformed Cultivar

Will the introduction of herbicide or insect tolerance genes to a cultivar increase the "weediness" of the plant? Characters that define a weed are debatable, but a general consensus of the traits that many weeds have was developed by Baker (1974). They include: (1) germination requirement fulfilled in many environments; (2) discontinuous germination and great longevity of seed; (3) rapid growth through vegetative phase to flowering; (4) continuous seed production for as long as growing conditions permit; (5) self-compatibility but not completely autogamous and apometic; (6) when cross-pollinated, unspecialized visitors or wind pollinated; (7) high seed output in favorable environments and some seed production in a wide range of environments; (8) adaption for short- and long-distance dispersal; (9) if perennial, vegetative production or regeneration from fragments and brittleness (so not easily removed from the ground); and (10) ability to compete interspecifically by special means (rosette formation and presence of allelochemicals). Not all weeds have all these characteristics. *G. hirsutum* cv. Stoneville 825 that was genetically transformed is not considered a weed and has few "weedy" traits. Introduction of these new genes into this cultivar has not significantly changed "weedy" characteristics. No change was noted with transformed cultivar in the number of seeds produced, germination characteristics of seeds, or the number of days from planting until first boll production or flowering.

Vertical Transfer of the Genes

It is apparent from the data shown in figure 8 that outcrossing from the transformed cultivar to other domestic cottons does and will occur. Of course, this kind of gene transfer happens in the field constantly. Because cotton producers purchase new seed every year, the cross pollination phenomenon does not have a significant impact on the quality or nature of seed produced in a field where cross pollination has occurred. Seeds from all transgenic cotton cultivars will still have to meet existing certification requirements for cotton seed production.

The noncultivated Gossypium spp. found in the southwestern U.S. and Hawaii are not considered weeds and introgression of the new genes into these species would not significantly increase any of the 9 characteristics of weeds (see above) unless selection pressure favors these characteristics. With regard to glyphosate-tolerant cotton, introgression of this trait into noncultivated Gossypium spp. would not expected to be highly favored in the absence of herbicide application. Herbicide application is only likely in agricultural settings not wild stands. With regard to the introduction of Bt-resistant plants in Hawaii: (1) Hawaii is not a cotton producing state. The great majority of cotton in the State is grown at experimental plots where cotton breeding programs operate. (2) There are no endangered or threatened lepidopteran insects listed in Federal Register in Hawaii (50 CFR 17.11 & 17.12). (3) In the case of the low probability of transfer of the transformed genes to native Hawaiian cotton (G. tomentosum) (assuming the exempted cultivar is used in a breeding program under our companies auspices), the risks to native lepidopteran insects would be no greater than those of using a nontransformed cotton line that is being developed for its resistance to lepidopteran insects by traditional breeding techniques. (4) Cotton breeders generally bag or clip the flowers when performing cross between plants. This significantly reducing the chances of visitation of the flowers by pollinators and thus reducing the likelihood of gene movement.

Several threatened or endangered butterflies are listed for California and Florida, two cotton producing states. There is no data on whether or not these species would be susceptible to this specific Bt protein. Based on the number of lepidopteran insects thus far tested for susceptibility to a specific Bt, most lepidopteran insects are not affected by a single Bt protein (Hofte and Whitely 1989). In addition, most of the listed species' historic ranges are not in cotton production areas, thus reducing the contact between the transformed plant and insect. In addition, these insects are not likely to feed or dependent on cotton. Many endangered lepidopteran are at risk for extinction from the loss of habitat. Also, the cultivar under review was developed specifically for the Mississippi Delta region of the southern U.S.

Horizontal Transfer of Genes

Genetic transfer across taxa of eukaryotes is suggested in only a few cases (Lewin 1982), and of these the only one

suggesting a transfer (excepting Agrobacterium) from unrelated taxa to higher plants is with the case of vertebrate hemoglobin and legume hemoglobin (Hyldig-Nielson et al. 1982). During the field testing of these plants there was no evidence of horizontal transfer of the transformed genes to adjacent nonsexually compatible plants.

VIII. **Statement of Grounds Unfavorable**

1. With respect to Bt-resistant cotton, bollworms and budworms resistant to Bt may develop. JPS Biotechnologies has introduced the Bt gene in cotton under the control of boll-specific promoter in an effort to slow the development of resistance.

2. With respect to the herbicide-tolerant cotton, the use of the herbicide glyphosate may increase if the transgenic cultivar is widely accepted by farmers. The increased use of glyphosate will be offset by the decreased use of organic arsenate based herbicides used in conjunction with other herbicides.

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